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(54) Title: IMMUNOTHERAPY BASED ON DENDRITIC CELLS

(57) Abstract: Dendritic cells are exposed to at least one bacterial strain in particular bacterial species present in the human commensal flora. The bacterial strain may be a Lactobacillus and/or Bifidobacterium and/or salmonella strain. The exposed dendritic cells or a formulation, pharmaceutical or vaccine comprising such dendritic cells may be used in the prevention and/or treatment of various diseases such as inflammatory diseases.

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"IMMUNOTHERAPY BASED ON DENDRITIC CELLS"**Introduction**

5 The invention relates to dendritic cells.

Dendritic cells are professional antigen presenting cells specialised for the initiation of T cell immunity. Physical contact between dendritic cells and T cells is required for the induction of T cell immunity. Dendritic cells activate antigen-specific
10 immune responses via two types of signalling steps. The first signal step involves the peptide-MHC/TCR interaction, while the second involves co-stimulatory molecules such as cell surface markers and cytokines.

Immune responses are characterised by their polarisation in the cytokines that are
15 produced. Dendritic cells produce an array of cytokines when they present antigens to T cells thus influencing the cytokine microenvironment and subsequent immune response.

Statements of Invention

20 The invention provides dendritic cells which have been exposed to at least one bacterial strain. The bacterial strain preferably has immunotherapeutic properties.

In a particularly preferred embodiment of the invention there is provided dendritic
25 cells which have been exposed to bacterial species present in the human commensal flora.

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In one embodiment the bacterial strain is a *Lactobacillus*, such as *Lactobacillus salivarius*, especially *Lactobacillus salivarius* subspecies *salivarius* and preferably *Lactobacillus salivarius* subspecies *salivarius* 433118.

5 In another embodiment the bacterial stain is a *Bifidobacterium*, such as *Bifidobacterium infantis*, especially *Bifidobacterium infantis* 35624.

In another embodiment the bacterial strain is salmonella, such as *Salmonella typhimurium*, especially *Salmonella typhimurium* UK1.

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The dendritic cells may be exposed to dead bacteria, or components or mutants thereof.

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The invention also provides an active derivative, fragment or mutant of dendritic cells of the invention.

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In a further aspect the invention provides a formulation comprising dendritic cells of the invention or an active derivative, fragment or mutant thereof. In particular the invention provides a pharmaceutical comprising dendritic cells of the invention or an active derivative, fragment or mutant thereof. Also provided is a vaccine comprising dendritic cells of the invention or an active derivative, fragment or mutant thereof.

25

In a further aspect the invention provides a method for activating dendritic cells comprising exposing dendritic cells to at least one bacterial strain. The bacterial strain may be a strain as defined above.

The dendritic cells of the invention or an active derivative, fragment or mutant thereof may have anti-inflammatory properties and/or anti-cancer properties and/or immuno-regulatory properties.

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The dendritic cells of the invention or an active derivative, fragment or mutant thereof may enhance immunological tolerance of specific antigens and/or activate cell-mediated immune responses to specific antigens and/or activate humoral immune responses to specific antigens.

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The dendritic cells of the invention or an active derivative, fragment or mutant thereof may stimulate regulatory T cell responses.

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The bacteria used in the invention may establish distinct cytokine networks by maturing naive dendritic cells.

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The dendritic cells of the invention or an active derivative, fragment or mutant thereof have potential therapeutic benefit in the following disease states: inflammatory disorders, immunodeficiency, inflammatory bowel disease, irritable bowel syndrome, cancer (particularly those of the gastrointestinal and immune systems), diarrhoeal disease, antibiotic associated diarrhoea, paediatric diarrhoea, appendicitis, autoimmune disorders, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, coeliac disease, diabetes mellitus, organ transplantation, bacterial infections, viral infections, fungal infections, periodontal disease, urogenital disease, sexually transmitted disease, HIV infection, HIV replication, HIV associated diarrhoea, surgical associated trauma, surgical-induced metastatic disease, sepsis, weight loss, anorexia, fever control, cachexia, wound healing, ulcers, gut barrier function, allergy, asthma, respiratory disorders, circulatory disorders, coronary heart disease, anaemia, disorders of the blood coagulation system, renal disease, disorders of the central nervous system, hepatic disease, ischaemia, nutritional disorders, osteoporosis, endocrine disorders, epidermal disorders, psoriasis and acne vulgaris.

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A deposit of *Lactobacillus salivarius* strain 433118 was made at the NCIMB on November 27, 1996 and accorded the accession number NCIMB 40829. The strain of *Lactobacillus salivarius* is described in WO-A-98/35014.

5 A deposit of *Bifidobacterium infantis* strain 35624 was made at the NCIMB on January 13, 1999 and accorded the accession number NCIMB 41003. The strain of *Bifidobacterium infantis* is described in WO-A-00/42168.

10 A strain of *Salmonella typhimurium* UK1 is described by Wilmes-Risenberg *et al.*, 1996, from whom a sample was obtained.

Detailed Description

15 This invention describes cytokine production by dendritic cells in response to different bacterial species, which influences the nature of subsequent T cell activation.

20 The microflora on mucosal surfaces are vast in number and complexity. Many hundreds of bacterial strains exist and account for approximately 90% of the cells found in the human body, the remainder of the cells being human. The vast majority of these bacterial strains do not cause disease and may actually provide the host with significant health benefits (e.g. *bifidobacteria* and *lactobacilli*). These bacterial strains are termed commensal organisms. Mechanism(s) exist whereby the immune system at mucosal surfaces can recognise commensal non-pathogenic flora as being
25 different to pathogenic organisms.

The human immune system plays a significant role in the aetiology and pathology of a vast range of human diseases. Hyper and hypo-immune responsiveness results in, or is a component of, the majority of disease states. One family of biological

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entities, termed cytokines, are particularly important to the control of immune processes. Perturbances of these delicate cytokine networks are being increasingly associated with many diseases. These diseases include but are not limited to inflammatory disorders, immunodeficiency, inflammatory bowel disease, irritable
5 bowel syndrome, cancer (particularly those of the gastrointestinal and immune systems), diarrhoeal disease, antibiotic associated diarrhoea, paediatric diarrhoea, appendicitis, autoimmune disorders, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, coeliac disease, diabetes mellitus, organ transplantation, bacterial infections, viral infections, fungal infections, periodontal disease, urogenital
10 disease, sexually transmitted disease, HIV infection, HIV replication, HIV associated diarrhoea, surgical associated trauma, surgical-induced metastatic disease, sepsis, weight loss, anorexia, fever control, cachexia, wound healing, ulcers, gut barrier function, allergy, asthma, respiratory disorders, circulatory disorders, coronary heart disease, anaemia, disorders of the blood coagulation system, renal disease, disorders
15 of the central nervous system, hepatic disease, ischaemia, nutritional disorders, osteoporosis, endocrine disorders, epidermal disorders, psoriasis and acne vulgaris.

The pre-programming of dendritic cells with bacteria would result in biologically active dendritic cells secreting regulatory cytokines. These regulatory cytokines
20 subsequently stimulate controlling immune responses. This invention describes the potential of different bacterial strains in customising dendritic cell phenotype and function. In this way customisation of disease specific therapies may be accomplished using a selection of bacterial strains.

25 Recognition of bacterial species by dendritic cells results in distinct patterns of cytokine production and immune responses. The cytokines produced by dendritic cells are secreted into the extracellular milieu. These cytokines deliver an informative signal to the T cell interacting specifically with the dendritic cell. In addition, secreted cytokines will also interact with neighbouring cells not specifically

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interacting with the dendritic cell. This "bystander" effect results in many different cell types being influenced by the cytokine network established by bacterial stimulated dendritic cells.

- 5 Aberrant presentation of antigen by dendritic cells results in many disease states, such as autoimmune disease (Drakesmith *et al.*, 2000). Thus, the re-establishment of immunological tolerance using appropriately primed dendritic cells is an attractive therapeutic option.
- 10 The immunomodulatory activity of dendritic cells has been demonstrated to have therapeutic potential in a number of model systems (Link *et al.*, 2001). Dendritic cell mediated tolerance has been achieved in animal models of experimental autoimmune encephalomyelitis and spontaneous diabetes (Huang *et al.*, 2000, Papaccio *et al.*, 2000). The *in vitro* transfection of dendritic cells with cytokines, 15 such as IL-10 and TGF β , enhances their suppressive potential (Thorbecke *et al.*, 2000) but gene therapy is still an inherently dangerous approach (Wilson, 2000). A more efficient and attractive approach would be to pulse dendritic cells *in vitro* with biologically active compounds which commit dendritic cells to an appropriate cytokine secretion pattern.
- 20 As the majority of cytokines may have both pro and anti-inflammatory activities, patterns or networks of cytokine release have been associated with different types of immune responses. The existence of T cells which differ in their pattern of cytokine secretion allows differentiation of inflammatory or immune responses into at least 25 three categories, cell mediated or humoral responses or Th3/Tr1 regulatory responses. Th1 responses are categorised by IFN γ , TNF β and IL-2 production leading to a cell-mediated response while Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13 resulting in a humoral response. Th3/Tr1 responses are characterised by T cell secretion of the regulatory cytokines IL-10 and TGF β . Differentiation of T cells

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into either network depends on the cytokine milieu in which the original antigen priming occurs (Seder et al., 1992). In addition, activation of T cells by dendritic cells leads to their differentiation into distinct populations of effector cells differing in their cytokine secretion pattern (Mosmann & Sad, 1996). These primary immune responses may also be influenced by a number of other cell types including $\gamma\delta$ T cells. Different types of stimulation may also direct this response such as immune complex deposition within inflammatory sites which increases IL-6 and IL-10 production and inhibits production of TNF α and IL-1 β thus influencing the Th1/Th2 balance. For successful elimination of some pathogens, the correct cytokine network needs to be established, such as the intracellular bacterium *Listeria monocytogenes* which elicits a Th1 response while the extracellular parasite *Nippostrongylus brasiliensis* requires a Th2 response. Each of these T cell subsets produce cytokines that are autocrine growth factors for that subset and promote differentiation of naive T cells into that subset (for review see Trinchieri et al., 1996). These two subsets also produce cytokines that cross-regulate each other's development and activity. IFN γ amplifies Th1 development and inhibits proliferation of Th2 T cells while IL-10 blocks Th1 activation. While the molecular events controlling Th1 and Th2 development are poorly understood, specific dendritic cell subclasses have been demonstrated to influence the elucidation of these different responses (Maldonado-Lopez et al., 1999). Tr1 cells have a profound suppressive effect on antigen-specific T cell responses mediated by secretion of IL-10 and TGF β (Groux et al., 1997) and cytokine independent mechanisms such as direct cell-cell contact. Stimulation of T cells by specific dendritic cells generates T cells that display the typical properties of Tr1 cells (Jonuleit et al., 2000).

The cytokine networks involved in immune responses are subject to a complex number of control pathways that normally result in restriction of cellular damage and eradication of the infectious organism. However, unregulated release of these cytokines can have damaging consequences. Incorrect Th1/ Th2 responses may

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contribute to the pathogenesis of certain diseases. For instance, the healing form of leprosy (tuberculoid lesion) is associated with a Th1 response while uncontrolled leprosy (lepromatous lesion) is associated with Th2 responses. Chronic inflammatory responses can lead to the death of the host. For instance, rats infected with the protazoan parasite *Trypanosoma brucei* become cachectic, develop anaemia and eventually die. Production of the proinflammatory cytokines has been associated with the pathogenesis of many disorders. In Langerhans cell histiocytosis, cytokines may be involved in some of the tissue damage seen with this disease (Kannourakis & Abbas, 1994). Rheumatoid arthritis is a chronic inflammatory disease of the synovial joints resulting in cartilage destruction and bone erosion (Kouskoff et al., 1996). High levels of proinflammatory cytokines have been detected from patients with rheumatoid arthritis and these levels could be associated with disease activity, altered energy metabolism and food intake (Roubenoff et al., 1994). In patients with sepsis, cardiovascular shock and organ dysfunction may be initiated by the production of proinflammatory cytokines stimulated by the infectious organism particularly in patients with cerebral malaria (Kwiatkowski et al., 1990). Certain alleles of polymorphic sites associated with TNF α production have been shown to predict patients with cerebral malaria (McGuire et al., 1994) and severe sepsis (Stuber et al., 1996) who will be most adversely affected. Genetic predisposition to increased TNF α production may also be associated with the development of autoimmune diseases such as diabetes and systemic lupus erythematosus. Inhibition of proinflammatory cytokine production has reduced the damage caused by many disease states. IL-1RA reduces the severity of diseases such as shock, lethal sepsis, inflammatory bowel disease, experimental arthritis and proliferation of human leukaemic cells (for review see Dinarello, 1992). Inhibition of TNF α in septic shock prevents the syndrome of shock and tissue injury despite persistent bacteraemia in animal models. Loss of the TNF receptor type I in knock-out mice protects against endotoxic shock (Pfeiffer et al., 1993). Anti-cytokine strategies in humans with sepsis have yielded disappointing results possibly due to

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complications such as the late administration of these factors after the initial inflammatory insult. However, studies involving neutralising TNF α antibodies in rheumatoid arthritis and Crohn's disease have had considerable success with significant reductions in disease activity being observed (Moreland et al., 1997, Stack et al., 1997). Inhibition of transcription factors, such as NF- κ B, which are responsible for intracellular signalling in the inflammatory response have been successful in reducing tissue damage in animals with chronic intestinal inflammation (Neurath et al., 1996). Moreover, adoptive transfer of T cells secreting IL-10 inhibited colitis in a murine model (Asserman *et al.*, 1999). Therefore, while the inflammatory response is critical to the defence and repair of host tissues, uncontrolled responses can result in significant tissue and organ damage and may result in the death of the host.

TGF β refers to a family of closely related molecules termed TGF β 1 to - β 5 (Roberts & Sporn, 1990). All are released from cells in a biologically inactive form due to their association with a latency protein which is believed to be a critical regulatory step. Three receptors have been identified for TGF β . Only two of these receptors transduce an intracellular signal suggesting a decoy function for the third receptor. Like the MIP family, TGF β also functions as a chemotactic factor for both monocytes and neutrophils. However, this cytokine has diverse effects as both pro and anti-inflammatory effects have been described. Aggregated platelets following vascular injury release TGF β resulting in inflammatory cell recruitment to the tissue. Activated monocytes and neutrophils synthesize TGF β further increasing cellular recruitment. Monocyte integrin expression is also enhanced by TGF β as is the induction of collagenase type IV which may aid movement through basement membranes into inflamed sites (Wahl et al., 1993). TGF β increases the expression of Fc γ RIII (CD16) which recognises antibody bound cells thereby increasing phagocytic activity. The production of inflammatory cytokines by monocytes can also be stimulated by TGF β . However, expression of IL-1 receptor antagonist (IL-

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1RA) is also increased suggesting that this cascade, in part, may be self regulating. TGF β is also important as a negative regulatory agent. It antagonises the effects of many of the inflammatory cytokines and inhibits the proliferation of thymocytes, B cells and haemopoietic stem cells. The activity of a number of cell types can be suppressed by TGF β including natural killer (NK) cells, cytotoxic T lymphocytes and lymphokine activated killer (LAK) cells. TGF β also has suppressive effects on the release of reactive oxygen and nitrogen intermediates by tissue macrophages (Ding et al., 1990). The immune inhibitory effects of TGF β can most clearly be observed in its effects on diseases such as experimental arthritis, multiple sclerosis and graft rejection. Through the stimulation of matrix protein production, TGF β may be important to wound healing which is also indicated by its chemotactic activity for fibroblasts (Roberts & Sporn, 1990). Therefore TGF β may have important functions with regard to resolution of the inflammatory response and promotion of healing within the inflammatory lesion.

IL-4, like IFN γ and IL-2, is a T cell derived cytokine. IL-4 has a molecular mass of 15 kDa and post-transcriptional glycosylation adds to this. While the IL-4 receptor can be membrane bound or secreted, they are coded for by separate genes unlike other soluble receptors which are derived by proteolysis of the membrane bound form. The effects of IL-4 seem to be species specific. This cytokine promotes murine macrophage proinflammatory cytokine synthesis while inhibiting production of the same cytokines in humans. IL-4 can enhance antigen-presentation (Aiello et al., 1990) and enhances T cell, B cell and mast cell proliferation (Arai et al., 1990). B cell class switching, MHC class II and Fc ϵ RII expression are all influenced by IL-4. IL-4 can also function as an anti-inflammatory agent. It can inhibit production of prostaglandins and collagenases (Corcoran et al., 1992). IL-4 may also promote apoptosis in stimulated monocytes (Mangan et al., 1992). IL-13 seems to be a cytokine that is functionally similar to IL-4, as both are T cell derived cytokines and

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both suppress monocyte proinflammatory cytokine production and affect surface antigen expression (Hart et al., 1995).

IL-10 is produced by T cells, B cells, monocytes and macrophages (De Waal Malefyt et al., 1991). This cytokine augments the proliferation and differentiation of B cells into antibody secreting cells (Go et al., 1990). IL-10 exhibits mostly anti-inflammatory activities. It up-regulates IL-1RA expression by monocytes and suppresses the majority of monocyte inflammatory activities. IL-10 inhibits monocyte production of cytokines, reactive oxygen and nitrogen intermediates, MHC class II expression, parasite killing and IL-10 production via a feed back mechanism (De Waal Malefyt et al., 1991). This cytokine has also been shown to block monocyte production of intestinal collagenase and type IV collagenase by interfering with a PGE₂-cAMP dependant pathway (Mertz et al., 1994) and therefore may be an important regulator of the connective tissue destruction seen in chronic inflammatory diseases.

IL-12 is a heterodimeric protein of 70 kD composed of two covalently linked chains of 35 kD and 40 kD. It is produced primarily by antigen presenting cells, such as macrophages, early in the inflammatory cascade. Intracellular bacteria stimulate the production of high levels of IL-12 (Ma et al., 1997). It is a potent inducer of IFN γ production and activator of natural killer cells. IL-12 is one of the key cytokines necessary for the generation of cell mediated, or Th1, immune responses primarily through its ability to prime cells for high IFN γ production (Schmitt et al., 1997). IL-12 induces the production of IL-10 which feedback inhibits IL-12 production thus restricting uncontrolled cytokine production. TGF- β also down-regulates IL-12 production (D'Andrea et al., 1995). IL-4 and IL-13 can have stimulatory or inhibitory effects on IL-12 production. Inhibition of IL-12 *in vivo* may have some therapeutic value in the treatment of Th1 associated inflammatory disorders, such as multiple sclerosis (Leonard et al., 1997).

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Interferon-gamma (IFN γ) is primarily a product of activated T lymphocytes and due to variable glycosylation it can be found ranging from 20 to 25 kDa in size. This cytokine synergizes with other cytokines resulting in a more potent stimulation of monocytes, macrophages, neutrophils and endothelial cells. IFN γ also amplifies lipopolysaccharide (LPS) induction of monocytes and macrophages by increasing cytokine production, increased reactive intermediate release, phagocytosis and cytotoxicity (Donnelly et al., 1990). IFN γ induces, or enhances the expression of major histocompatibility complex class II (MHC class II) antigens on monocytic cells and cells of epithelial, endothelial and connective tissue origin (Arai et al., 1990). This allows for greater presentation of antigen to the immune system from cells within inflamed tissues. IFN γ may also have anti-inflammatory effects. This cytokine inhibits phospholipase A₂, thereby decreasing monocyte production of PGE₂ and collagenase (Wahl et al., 1990). IFN γ may also modulate monocyte and macrophage receptor expression for TGF β , TNF α and C5a thereby contributing to the anti-inflammatory nature of this cytokine. Probiotic stimulation of this cytokine would have variable effects *in vivo* depending on the current inflammatory state of the host, stimulation of other cytokines and the route of administration.

TNF α is a proinflammatory cytokine which mediates many of the local and systemic effects seen during an inflammatory response. This cytokine is primarily a monocyte or macrophage derived product but other cell types including lymphocytes, neutrophils, NK cells, mast cells, astrocytes, epithelial cells (Neale et al., 1995) endothelial cells and smooth muscle cells can also synthesise TNF α . TNF α is synthesised as a prohormone and following processing the mature 17.5 kDa species can be observed. Purified TNF α has been observed as dimers, trimers and pentamers with the trimeric form postulated to be the active form *in vivo*. Three receptors have been identified for TNF α . A soluble receptor seems to function as a TNF α inhibitor while two membrane bound forms have been identified with molecular sizes of 60 and 80 kDa respectively (Schall et al., 1990). Local TNF α production at

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inflammatory sites can be induced with endotoxin and the glucocorticoid dexamethasone inhibits cytokine production. TNF α production results in the stimulation of many cell types. Significant anti-viral effects could be observed in TNF α treated cell lines and the IFNs synergise with TNF α enhancing this effect (Wong & Goeddel, 1986). Endothelial cells are stimulated to produce procoagulant activity, expression of adhesion molecules, IL-1, hematopoietic growth factors, platelet activating factor (PAF) and arachidonic acid metabolites. TNF α stimulates neutrophil adherence, phagocytosis, degranulation, reactive oxygen intermediate production and may influence cellular migration (Livingston et al., 1989). Leucocyte synthesis of GM-CSF, TGF β , IL-1, IL-6, PGE₂ and TNF α itself can all be stimulated upon TNF α administration (Cicco et al., 1990). Programmed cell death (apoptosis) can be delayed in monocytes (Mangan et al., 1991) while effects on fibroblasts include the promotion of chemotaxis and IL-6, PGE₂ and collagenase synthesis. While local TNF α production promotes wound healing and immune responses, the dis-regulated systemic release of TNF α can be severely toxic with effects such as cachexia, fever and acute phase protein production being observed (Dinarello et al., 1988).

Dendritic cell therapies for the treatment of cancer have achieved some success. However, a number of mechanisms have been described which allow tumour cells to escape immunological destruction. Although tumours express antigenic determinants they are not eliminated by the host's immune system. Either the antigens are not being presented efficiently and consequently do not elicit a powerful enough immune response or there is continuous selection, ongoing in the cancer patient, for tumour cells that can evade immune recognition. For efficient antigen presentation, the antigen needs to be expressed on professional antigen presenting cells (APC) through MHC class II to CD4 helper T cells and through MHC class I, on tumour cells, to CD8 cytotoxic T cells. This process also requires the interaction of co-stimulatory molecules such as B7-CD28, CD70-CD27 and CD40-CD40

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complexes with appropriate cytokine production. In patients with cancer this system does not seem to operate effectively and this failure could be due to a number of reasons. The down-regulation of MHC molecules on tumour cells has been well described (Restifo et al., 1993) and the antigen processing machinery of the tumour cells may be defective (Cromme et al., 1994). Tumour cell antigen presentation in the absence of costimulatory molecules may induce tolerance as demonstrated by animal experiments where immune responses were amplified when B7-1 or B7-2 were expressed on tumour cells (Shu et al., 1997). The development of antigen-specific T cell anergy may be an early event in the tumour-bearing host, suggesting that tolerance to tumour antigens may represent a significant barrier to immunotherapy (Staveley-O'Carroll et al., 1998). However, tolerance to certain tumour specific antigens, such as carcinoembryonic antigen (CEA), may be broken by immunisation with a recombinant virus expressing CEA (Tsang et al., 1995). T cells that have been repeatedly activated express CD95 (Fas) on their surface and are therefore sensitive to killing by tumour cells expressing Fas ligand (Hahne et al., 1996). Thus, tumour cells could be inducing apoptosis in the T cells that are recognising them as foreign.

At initial stages of tumour growth in a murine model, anti-tumour immune responses are induced but with increasing tumour burden a generalised immunosuppression becomes evident (Gahan et al., 1997). Patients with advanced cancer are frequently found to exhibit impaired immune responses and a variety of immuno-suppressive mechanisms have been described. Usually, immuno-suppression is confined to the tumour region except for a few cases of advanced disease (O' Sullivan et al., 1996). Tumour derived products may interfere with the local immune response. Immuno-suppressive cytokines produced by tumour cells include transforming growth factor β (TGF β), interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF). These cytokines have a number of suppressive effects on tumour infiltrating lymphocyte function suggesting that potent immuno-suppressive mechanisms may

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be at work within the tumour bed (Spellman et al. 1996). IL-10 is also a potent inhibitor of tumour cytotoxicity by monocytes and alveolar macrophages. Prostaglandin production in the vicinity of the tumour inhibits IL-2 induced T cell proliferation while tumour cell induction of nitric oxide production decreased mononuclear cell proliferation. Immune suppressive factors in tumour bearing hosts may induce lymphoid apoptosis (O Mahony et al., 1993). Soluble antigens shed by tumour cells may interfere with immune responses to tumours. Host CD4 T cells may play a role in tumour immune evasion as induction of Th2 responses may inhibit Th1 cell-mediated responses which are thought to be important for anti-tumour immunity.

Vaccination with dendritic cells has been demonstrated to break immunological tolerance of tumour cells and induce tumour lysis via Th1 type responses. However, strategies to date have focussed on identifying specific tumour antigens and defining antigenic peptides that bind to the particular MHC alleles expressed by each patient (Nestle *et al.*, 1998). A more general approach would be to use dendritic cells previously exposed to specific bacterial stimuli. Exposure to the bacterial strains outlined in this invention would activate dendritic cells in a manner appropriate for stimulation of anti-tumour immune responses irrespective of the antigens present. Dendritic cells could also be pulsed with tumour antigens *in vitro* or *in vivo*. Cytokine production by activated dendritic cells in the tumour microenvironment would promote anti-tumour immune responses.

The majority of pathogenic organisms gain entry via mucosal surfaces. Efficient vaccination of these sites protects against invasion by a particular infectious agent. Oral vaccination strategies have concentrated, to date, on the use of attenuated live pathogenic organisms or purified encapsulated antigens (Walker, 1994). However, vaccination with antigen-pulsed dendritic cells, previously exposed to biotherapeutic

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compounds, such as bacteria, could result in a more effective protective immune response.

5 The invention will be more clearly understood from the following examples.

Example 1. Cytokine profiles of murine bone marrow derived dendritic cells stimulated with probiotic and pathogenic bacterial strains.

10

Method

Mice were sacrificed by cervical dislocation and long bones excised. All adherent connective and muscle tissue was removed. Bones were sterilized by a rapid immersion in 70% ethanol and rinse in sterile PBS. The marrow was flushed repeatedly from the bones using 3ml HBSS per bone. The cells were pelleted and resuspended in sterile water to lyse RBCs. The cells were immediately resuspended in HBSS and centrifuged again. The cells were resuspended in 3ml RPMI 1640 plus 150µl of each antibody directed against B cells (ATCC, TIB229), anti Ia (ATCC, TIB150), anti-CD8 (ATCC, TIB 207) and anti-CD4 (ATCC, TIB 146). Following the addition of 50µl of complement (Sigma) the cells were incubated @37°C for 1 hour. Cells were washed twice and resuspended in 36ml RPMI. 3ml of cells per well were plated in a 12 well plate (Costar) and incubated overnight @37°C. The non-adherent cells were removed and a new 12 well plate (Costar) plated. 4ng/ml IL-4 (R&D Systems) and 2ng/ml GM-CSF (R&D Systems) were added. The cells were allowed to mature for 7-8 days @37°C. 1.5ml of fresh medium was added to each well on day 4. Following maturation of these dendritic cells, cells were scraped off the plates, pooled and counted. Cells were typically re-plated at 5×10^5 /ml in one ml in a 24 well plate (Costar). Cells were stimulated with 10µg/ml LPS (Sigma, L3024), bacteria ($10^2 - 10^6$ cells/ml) or

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remained non-stimulated. Following 24 hours of culture, supernatants were harvested, aliquoted and stored at -20°C . Culture supernatants were examined for IL-4, IL-10, IL-12, $\text{IFN}\gamma$, $\text{TGF}\beta$ and $\text{TNF}\alpha$ levels using ELISAs (Pharmingen).

5 Results

IL-4, IL-10, IL-12, $\text{IFN}\gamma$, $\text{TNF}\alpha$ and $\text{TGF}\beta$ from dendritic cell culture supernatants were quantified following exposure to LPS, *Bifidobacterium* 35624 or *Salmonella typhimurium* (Fig. 1). LPS stimulated the production of IL-10, IL-12, $\text{TNF}\alpha$ and $\text{TGF}\beta$ compared to control cultures. *Bifidobacterium* 35624 enhanced the production
10 of IL-10 and $\text{TGF}\beta$, with a low level of $\text{TNF}\alpha$ stimulation. *Salmonella typhimurium* enhanced the production of IL-4, IL-10, IL-12, $\text{IFN}\gamma$ and $\text{TNF}\alpha$, with a low level of $\text{TGF}\beta$ stimulation.

**Example 2. Cytokine profiles of murine gastrointestinal tract derived dendritic
15 cells stimulated with probiotic and pathogenic bacterial strains.**

Method

Mice were anaesthetised and sacrificed by cervical dislocation (n=4). The gastrointestinal tract was removed, opened longitudinally and surface sterilised by a
20 rapid immersion in 70% ethanol. The gastrointestinal tissue was incubated for 20 minutes shaking @ 37°C in 25mls HBSS containing DTT (0.145 mg/ml) and EDTA (0.37 mg/ml). Supernatants were decanted and the remaining tissue was incubated for 90 minutes shaking @ 37° in 25mls RPMI containing collagenase (0.15 mg/ml) and DNase (0.1 mg/ml). Supernatants were decanted and low speed centrifugation
25 removed tissue debris and clumps of cells. Following high speed centrifugation, single cells were isolated. These cells were incubated with 10% normal mouse serum and magnetic CD11c beads for 15 minutes @ 4°C . Cells were passed through a magnetic column twice in order to enrich for CD11c positive cells. These cells

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were incubated for 24 hours with *Lactobacillus* 433118, or *Salmonella typhimurium*, or LPS or remained non-stimulated as a negative control. Supernatants were collected and stored at -70°C . IL-10 and IL-12 cytokine levels were quantified using ELISAs (Pharmingen).

5

Results

Gut derived dendritic cells were incubated with a variety of bacterial stimuli (Fig. 2). Control cultures spontaneously produced IL-10 and IL-12. Stimulation with LPS enhanced IL-10 production but decreased IL-12 levels. Co-incubation with the *Salmonella* strain did not significantly alter IL-10 levels but did result in significant stimulation of IL-12 production. The probiotic 433118 enhanced the production of IL-10 and reduced IL-12 secretion.

10

Example 3. Modulation of cytokine production in bacterial stimulated, human mesenteric lymph node derived, dendritic cells.

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Method

Following surgical removal of human colons, mesenteric lymph nodes were removed. Mesenteric lymph node cells were isolated using density gradient centrifugation and dendritic cells were purified using magnetic bead isolation. Dendritic cells were stimulated in vitro with *Bifidobacterium* 35624, *Lactobacillus salivarius* 433118 or *Salmonella typhimurium* for 3 days. Supernatants were removed and cytokines were quantified using ELISAs.

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Results

Dendritic cells stimulated with different bacteria secreted distinct cytokine profiles (Fig. 3). *Bifidobacterium* 35624 and *Lactobacillus* 433118 stimulated the production of Th2 and Th3 regulatory cytokines while *Salmonella* stimulated the production of Th1 regulatory cytokines. *Lactobacillus* 433118 was also found to stimulate the production of Th2 and Th3 regulatory cytokines (results not shown).

Dendritic cells isolated from both mice and humans react in a similar manner to bacterial stimulation. Thus, the use of murine models to examine the therapeutic potential of bacterial stimulated dendritic cells is appropriate.

Example 4. Systemic modulation of immune-responsiveness following oral consumption of probiotic bacteria.

Method

A feeding trial involving 3 groups (n=10/group) of IL-10 knockout mice was performed. Each group consumed the probiotic *Lactobacillus* 433118, or *Bifidobacterium* 35624 or a placebo product for 19 weeks. At this time point all mice were sacrificed by cervical dislocation. The gastrointestinal tract was removed, examined and graded histologically for inflammatory activity. Whole spleens were aseptically removed and the mononuclear cell population was isolated using mechanical disruption and density gradient centrifugation. 1×10^6 spleen cells were stimulated *in vitro* with the probiotic 433118, or 35624, or the proinflammatory bacterium *Salmonella typhimurium* UK1, or remain non-stimulated as negative controls. Following 72 hours of incubation, supernatants were harvested and stored at -70°C . ELISAs were subsequently performed in order to quantify IL-12, $\text{IFN}\gamma$, $\text{TNF}\alpha$ and $\text{TGF}\beta$ cytokine levels (Pharmingen). Statistical analysis of group differences was performed using ANOVA analysis of variance.

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Results

Significant numbers of both probiotic strains were recovered over the feeding trial period. *Bifidobacterium* 35624 was recovered at approximately 1×10^5 CFU/g while *Lactobacillus* 433118 was recovered at approximately 1×10^7 CFU/g. Gastrointestinal inflammatory scores were significantly reduced for the mice consuming either probiotic compared to the control group (Fig. 4). Following the *in vitro* stimulation of murine spleenocytes, significant decreases were observed for TNF α (Fig. 5), IL-12 (Fig. 6) and IFN γ (Fig. 7) levels, but not TGF β levels (Fig. 8).

This study demonstrates that an immunomodulatory signal was transmitted from the gastrointestinal tract, following consumption of the probiotic strains 433118 and 35624, to the spleen. Interaction of the consumed bacterial strains in the gastrointestinal tract with dendritic cells and subsequent migration of these dendritic cells to distant sites, such as the spleen, resulted in a significant alteration of cytokine production at these sites. It can be envisaged that administration of bacterial treated dendritic cells alone would also deliver this therapeutic immunological signal.

Example 5. Systemic anti-inflammatory effects of *Lactobacillus* 433118 and *Bifidobacterium* 35624

Method

DBA1 mice were fed with *Lactobacillus* 433118 or *Bifidobacterium* 35624 (n=10 per group). Following probiotic feeding, rheumatoid arthritis was induced following collagen tail vein injection in groups 2 - 4. Inflammatory arthritis was measured by quantifying footpaw swelling with callipers.

Group 1: Healthy mice – no interventions

Group 2: Placebo feed

Group 3: *Lactobacillus* 433118

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Group 4: *Bifidobacterium* 35624

Results

Footpaw swelling was measured for all four paws in duplicate for each mouse. A statistically significant reduction in foot paw swelling was observed in mice consuming *Bifidobacterium* 35624 but not with *Lactobacillus* 433118 (Fig. 9). This study demonstrates that this probiotic bacterium induces immune-regulatory cells and mediators outside the gastrointestinal tract. The most important cellular mediator of these effects are dendritic cells and the regulatory T cells stimulated by dendritic cells.

Example 6. Anti-cancer properties of bacterial stimulated dendritic cells.

Method

Bone marrow derived dendritic cells were isolated from Balb/c mice using magnetic bead isolation and cultured for 7-8 days *in vitro* in the presence of GM-CSF and IL-4. Following expansion and maturation, dendritic cells were incubated with or without *Bifidobacterium infantis* 35624 for 90 minutes, in addition to co-incubation with JBS tumour cell lysates. JBS tumour cells survive and proliferate rapidly in immune competent balb/c mice. Balb/c mice were injected subcutaneous with:

Group 1: 1×10^5 dendritic cells pre-incubated with JBS lysates alone;
Group 2: 1×10^5 dendritic cells pre-incubated with JBS lysates plus *Bifidobacterium* 35624.

The balb/c mice were injected on two separate occasions using the procedure outlined above (n=8 mice per group). Concurrently, all mice were injected with live

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JBS tumour cells. Two weeks following tumour inoculation, all mice were sacrificed by cervical dislocation, tumours excised and weighed.

Results

- 5 The mean tumour volume was decreased in mice vaccinated with *Bifidobacterium* stimulated dendritic cells compared to mice vaccinated by dendritic cells alone (Fig. 10). Thus, adoptive transfer of *Bifidobacterium* 35624 activated dendritic cells can restrict the rate of JBS tumour growth.
- 10 The complexity and intimacy of the interactions that occur between bacteria and the host eukaryotic cells have only begun to be elucidated. The nature of these interactions creates a major paradox. The human being has a vast number of bacteria living on or in the host, representing 90% of all cells found in the body. These bacteria constitute the commensal flora found on all mucosal and epidermal
- 15 structures. Populations of these bacteria vary between the oral cavity, gastrointestinal tract, urogenital tract and the skin surface. The immune system recognizes the presence of these foreign microbes and therefore would be expected to launch significant immune responses resulting in chronic inflammatory lesions at these sites. However, this is not the case. The commensal microflora and the host
- 20 systems exist in a finely balanced environment whereby bacterial communities thrive and host tissues are not damaged by their own immune system. Evolution has selected for individuals whose immune system tolerates the presence of the non-pathogenic commensal flora while being able to react rapidly to the presence of pathogenic microbes. While the mechanisms underlying this immunological
- 25 perception are currently unclear, dendritic cells appear to have the ability to secrete different cytokines depending on the specific bacterial stimulus. As the dendritic cell provides the link between innate and adaptive immune responses, it is perfectly poised to control the nature of this response. Ultimately, the decision to attack or tolerate specific antigens may reside with the dendritic cell.

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This invention is not limited to dendritic cells isolated only in the manner as described herein, but applies to dendritic cells isolated using any technology and derived from any body compartment or tissue.

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This invention describes the cytokine network established due to stimulation of dendritic cells with *Lactobacillus*, *Bifidobacterium* and *Salmonella* species. However, this technology can be applied to all bacterial types and should not be limited to these bacterial strains alone. It is expected that stimulation of dendritic
10 cells with different bacterial species will result in dendritic cells with different cytokine profiles. These different immuno-therapeutic properties are applicable to a wide range of disease states.

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It is unknown whether the bacterial strains are required to exert an immuno-modulatory effect or if individual active components of the bacterial strains can be utilised alone. Proinflammatory components of certain bacterial strains have been identified. The proinflammatory effects of gram-negative bacteria are mediated by liposaccharide (LPS). LPS alone induces a proinflammatory network, partially due to LPS binding to the CD14 receptor on monocytes. It is assumed that components
20 of probiotic bacteria possess anti-inflammatory activity, due to the effects of the whole cells. Upon isolation of these components, pharmaceutical grade manipulation is anticipated. Therefore the term bacterial strain as used in this specification refers to active components thereof.

25

The general use of the bacterial strains is in the form of viable cells. However, it can also be extended to non-viable cells such as killed cultures or compositions containing beneficial factors expressed by the bacterial strains. This could include thermally killed micro-organisms or micro-organisms killed by exposure to altered pH or subjection to pressure. With non-viable cells product preparation is simpler,

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cells may be incorporated easily into pharmaceuticals and storage requirements are much less limited than viable cells. *Lactobacillus casei* YIT 9018 offers an example of the effective use of heat killed cells as a method for the treatment and/or prevention of tumour growth as described in US Patent No. US4347240.

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The specific application of bacterial activated dendritic cells for the treatment of human disease will depend on the disease state being treated. Dendritic cells can be isolated from all types of human tissue, including peripheral blood, mucosal sites, etc. It is envisaged that tissue will be isolated from a patient by a physician.

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Following removal of patient tissue, dendritic cells are purified, under sterile conditions, using antibody-labelling techniques (such as magnetic bead isolation). Dendritic cells may be cultured *in vitro* with cytokines and subsequently activated by bacterial cells, or can be activated immediately following purification by bacterial cells. Bacterial activated dendritic cells are administered back to the same patient

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from whom they were first isolated. The route of administration may be parenteral or enteral, including subcutaneous injection, intramuscular injection, intraperitoneal injection, intravenous injection, intravenous drip, nasal spray, oral consumption in enteric coated capsules, etc. Dendritic cells may be administered in a saline or nutrient solution, or can be administered with an adjuvant. For treatment of cancer

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patients, dendritic cells can be co-administered with tumour cells, preferably derived from the same patient. In other disease states, dendritic cells may be co-administered with antigens associated with disease pathology, such as myelin basic protein (i.e. multiple sclerosis). It is anticipated that dendritic cells may be administered at greater than 1×10^5 cells per patient and that treatment can be repeated as required.

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The invention is not limited to the embodiments hereinbefore described which may be varied in detail.

- 25 -

References

Wilmes-Riesenberg M.R., Bearson B., Foster J.W. & Curtiss R. Role of the acid tolerance response in virulence of *Salmonella typhimurium*. Infect. Immun., 1996:1085-92.

5

Seder RA, Paul WE, Davis MM, Fazekas de St Groth B. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J Exp Med 1992 Oct 1;176(4):1091-8.

10

Mosmann T.R. & Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today, 1996; 17:138-46.

15

Trinchieri G, Peritt D, Gerosa F. Acute induction and priming for cytokine production in lymphocytes. Cytokine Growth Factor Rev 1996 Aug;7(2):123-32.

20

Maldonado-Lopez R., De Smedt T., Michel P., Godfroid J., Pajak B., Heirman C., Thielemans K., Leo O., Urbain J. & Moser M. CD8 α ⁺ and CD8 α ⁻ subclasses of dendritic cells direct the development of distinct T helper cells in vivo. J. Exp. Med., 1999; 189:587-92.

25

Groux H., O' Garra A., Bigler M., Rouleau M., Antonenko S., de Vries J.E. & Roncarolo M.G. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature, 1997; 389:737-42.

30

Jonuleit H., Schmitt E., Schuler G., Knop J. & Enk A.H. Induction of interleukin 10-producing, nonproliferating CD4+ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J. Exp. Med., 2000; 192:1213-22.

- 26 -

Kannourakis G, Abbas A. The role of cytokines in the pathogenesis of Langerhans cell histiocytosis. *Br J Cancer Suppl* 1994 Sep;23:S37-40.

5 Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. *Cell* 1996 Nov 29;87(5):811-22.

10 Roubenoff R, Roubenoff RA, Cannon JG, Kehayias JJ, Zhuang H, Dawson-Hughes B, Dinarello CA, Rosenberg IH. Rheumatoid cachexia: cytokine-driven hypermetabolism accompanying reduced body cell mass in chronic inflammation. *J Clin Invest* 1994 Jun;93(6):2379-86.

15 Kwiatkowski D, Hill AV, Sambou I, Twumasi P, Castracane J, Manogue KR, Cerami A, Brewster DR, Greenwood BM. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet* 1990 Nov 17;336(8725):1201-4.

20 McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature* 1994 Oct 6;371(6497):508-10.

25 Stuber F, Petersen M, Bokelmann F, Schade U. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. *Crit Care Med* 1996 Mar;24(3):381-4.

Dinarello CA. The role of interleukin-1 in host responses to infectious diseases. *Infect Agents Dis* 1992 Oct;1(5):227-36.

- 27 -

Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 1993 May 7;73(3):457-67.

5

Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, Ettlinger RE, Cohen S, Koopman WJ, Mohler K, Widmer MB, Blosch CM. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N Engl J Med* 1997 Jul 17;337(3):141-7.

10

Stack WA, Mann SD, Roy AJ, Heath P, Sopwith M, Freeman J, Holmes G, Long R, Forbes A, Kamm MA. Randomised controlled trial of CDP571 antibody to tumour necrosis factor-alpha in Crohn's disease. *Lancet* 1997 Feb 22;349(9051):521-4.

15

Neurath MF, Pettersson S, Meyer zum Buschenfelde KH, Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med* 1996 Sep;2(9):998-1004.

20

Asserman C., Mauze S., Leach M.W., Coffman R.L. & Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.*, 1999; 190:995-1003.

25

Drakesmith H., Chain B. & Beverly B. How can dendritic cells cause autoimmune disease? *Immunol. Today*, 2000; 21:214-7.

Link H., Huang Y.-M., Masterman T. & Xiao B.-G. Vaccination with autologous dendritic cells: from experimental autoimmune encephalomyelitis to multiple sclerosis. *J. Neuroimmunol.*, 2001; 114:1-7.

- 28 -

- 5 Huang Y.M., Yang J.S., Xu L.Y., Link H. & Xiao B.G. Autoantigen-pulsed dendritic cells induce tolerance to experimental allergic encephalomyelitis in Lewis rats. *Clin. Exp. Immunol.*, 2000; 122, 437-44.
- 10 Papaccio G., Nicoletti F., Pisanti F.A., Bendtzen K. & Galdieri M. Prevention of spontaneous autoimmune diabetes in NOD mice by transferring in vitro antigen-pulsed syngeneic dendritic cells. *Endocrinology*, 2000; 141:1500-5.
- 15 Thorbecke G.J., Umetsu D.T., deKruyff R.H., Hansen G., Chen L.Z. & Hochwald G.M. When engineered to produce TGF- β 1, antigen specific T cells down regulate Th1 cell-mediated autoimmune and Th2 cell-mediated allergic inflammatory processes. *Cytokine Growth Factor Rev.*, 2000; 11:89-96.
- 20 Wilson J.M. Researchers and regulators reflect on first gene therapy death. *Nat. Med.*, 2000; 6:6.
- 25 Restifo NP, Kawakami Y, Marincola F, Shamamian P, Taggarse A, Esquivel F, Rosenberg SA. Molecular mechanisms used by tumors to escape immune recognition: immunogenetherapy and the cell biology of major histocompatibility complex class I. *J Immunother* 1993 Oct;14(3):182-90.
- 30 Cromme FV, Airey J, Heemels MT, Ploegh HL, Keating PJ, Stern PL, Meijer CJ, Walboomers JM. Loss of transporter protein, encoded by the TAP-1 gene, is highly correlated with loss of HLA expression in cervical carcinomas. *J Exp Med* 1994 Jan 1;179(1):335-40.

- 29 -

Shu S, Plautz GE, Krauss JC, Chang AE. Tumor immunology. JAMA 1997 Dec 10;278(22):1972-81.

5 Staveley-O'Carroll K, Sotomayor E, Montgomery J, Borrello I, Hwang L, Fein S, Pardoll D, Levitsky H. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. Proc Natl Acad Sci U S A 1998 Feb 3;95(3):1178-83.

10 Tsang KY, Zaremba S, Nieroda CA, Zhu MZ, Hamilton JM, Schlom J. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J Natl Cancer Inst 1995 Jul 5;87(13):982-90.

15 Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, Schneider P, Bornand T, Fontana A, Lienard D, Cerottini J, Tschopp J. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. Science 1996 Nov 22;274(5291):1363-6.

20 Gahan CG, Barrett JR, O'Brien MG, O'Sullivan GC, Shanahan F, Collins JK. Innate resistance to *Listeria monocytogenes* in tumor-bearing mice. J Leukoc Biol 1997 Dec;62(6):726-32.

25 O'Sullivan GC, Corbett AR, Shanahan F, Collins JK. Regional immunosuppression in esophageal squamous cancer: evidence from functional studies with matched lymph nodes. J Immunol 1996 Nov 15;157(10):4717-20.

Spellman JE, Gollnick SO, Zhang PJ, Tomasi TB. Cytokine production by human soft tissue sarcomas: implications for immunosuppression within the tumour bed. Surg Oncol 1996 Oct-Dec;5(5-6):237-44.

- 30 -

5 O'Mahony AM, O'Sullivan GC, O'Connell J, Cotter TG, Collins JK. An immune suppressive factor derived from esophageal squamous carcinoma induces apoptosis in normal and transformed cells of lymphoid lineage. J Immunol 1993 Nov 1;151(9):4847-56.

10 Nestle F., Alijagic S., Gilliet M., Sun Y., Grabbe S., Dummer R., Burg G. & Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., 1998; 2:328-32.

Walker R.J. *Vaccine*, 1994, 12, 387. Walker R.J. *Vaccine*, 1994, 12, 387.

15 Roberts AB, Flanders KC, Heine UI, Jakowlew S, Kondaiah P, Kim SJ, Sporn MB. Transforming growth factor-beta: multifunctional regulator of differentiation and development. Philos Trans R Soc Lond B Biol Sci 1990 Mar 12;327(1239):145-54.

20 Wahl SM, Allen JB, Weeks BS, Wong HL, Klotman PE. Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocytes. Proc Natl Acad Sci U S A 1993 May 15;90(10):4577-81.

25 Ding A, Nathan CF, Graycar J, Derynck R, Stuehr DJ, Srimal S. Macrophage deactivating factor and transforming growth factors-beta 1 -beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. J Immunol 1990 Aug 1;145(3):940-4.

Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: coordinators of immune and inflammatory responses. Annu Rev Biochem 1990;59:783-836.

- 31 -

- Aiello FB, Longo DL, Overton R, Takacs L, Durum SK. A role for cytokines in antigen presentation: IL-1 and IL-4 induce accessory functions of antigen-presenting cells. *J Immunol* 1990 Apr 1;144(7):2572-81.
- 5 Corcoran ML, Stetler-Stevenson WG, Brown PD, Wahl LM. Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92-kDa type IV collagenase/gelatinase production by human monocytes. *J Biol Chem* 1992 Jan 5;267(1):515-9.
- 10 Mangan DF, Welch GR, Wahl SM. Lipopolysaccharide, tumor necrosis factor-alpha, and IL-1 beta prevent programmed cell death (apoptosis) in human peripheral blood monocytes. *J Immunol* 1991 Mar 1;146(5):1541-6.
- 15 Hart PH, Ahern MJ, Smith MD, Finlay-Jones JJ. Regulatory effects of IL-13 on synovial fluid macrophages and blood monocytes from patients with inflammatory arthritis. *Clin Exp Immunol* 1995 Mar;99(3):331-7.
- 20 de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* 1991 Oct 1;174(4):915-24.
- 25 Go NF, Castle BE, Barrett R, Kastelein R, Dang W, Mosmann TR, Moore KW, Howard M. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med* 1990 Dec 1;172(6):1625-31.

- 32 -

Mertz PM, DeWitt DL, Stetler-Stevenson WG, Wahl LM. Interleukin 10 suppression of monocyte prostaglandin H synthase-2. Mechanism of inhibition of prostaglandin-dependent matrix metalloproteinase production. *J Biol Chem* 1994 Aug 19;269(33):21322-9.

5

Ma X, Aste-Amezaga M, Gri G, Gerosa F, Trinchieri G. Immunomodulatory functions and molecular regulation of IL-12. *Chem Immunol* 1997;68:1-22.

10

Schmitt E, Rude E, Germann T. The immunostimulatory function of IL-12 in T-helper cell development and its regulation by TGF-beta, IFN-gamma and IL-4. *Chem Immunol* 1997;68:70-85.

15

D'Andrea A, Ma X, Aste-Amezaga M, Paganin C, Trinchieri G. Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production. *J Exp Med* 1995 Feb 1;181(2):537-46.

20

Leonard JP, Waldburger KE, Schaub RG, Smith T, Hewson AK, Cuzner ML, Goldman SJ. Regulation of the inflammatory response in animal models of multiple sclerosis by interleukin-12. *Crit Rev Immunol* 1997;17(5-6):545-53.

25

Donnelly RP, Fenton MJ, Finbloom DS, Gerrard TL. Differential regulation of IL-1 production in human monocytes by IFN-gamma and IL-4. *J Immunol* 1990 Jul 15;145(2):569-75.

Wahl LM, Corcoran ME, Mergenhagen SE, Finbloom DS. Inhibition of phospholipase activity in human monocytes by IFN-gamma blocks endogenous prostaglandin E2-dependent collagenase production. *J Immunol* 1990 May 1;144(9):3518-22.

- 33 -

- Neale TJ, Ruger BM, Macaulay H, Dunbar PR, Hasan Q, Bourke A, Murray-McIntosh RP, Kitching AR. Tumor necrosis factor-alpha is expressed by glomerular visceral epithelial cells in human membranous nephropathy. *Am J Pathol* 1995 Jun;146(6):1444-54.
- 5 Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GH, Gatanaga T, Granger GA, Lentz R, Raab H, et al. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell* 1990 Apr 20;61(2):361-70.
- 10 Wong GH, Goeddel DV. Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. *Nature* 1986 Oct 30-Nov 5;323(6091):819-22.
- 15 Livingston DH, Appel SH, Sonnenfeld G, Malangoni MA. The effect of tumor necrosis factor-alpha and interferon-gamma on neutrophil function. *J Surg Res* 1989 Apr;46(4):322-6.
- 20 Cicco NA, Lindemann A, Content J, Vandenbussche P, Lubbert M, Gauss J, Mertelsmann R, Herrmann F. Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. *Blood* 1990 May 15;75(10):2049-52.
- 25 Mangan DF, Welch GR, Wahl SM. Lipopolysaccharide, tumor necrosis factor-alpha, and IL-1 beta prevent programmed cell death (apoptosis) in human peripheral blood monocytes. *J Immunol* 1991 Mar 1;146(5):1541-6.
- Dinareello CA, Cannon JG, Wolff SM. New concepts on the pathogenesis of fever. *Rev Infect Dis* 1988 Jan-Feb;10(1):168-89.

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CLAIMS

1. Dendritic cells which have been exposed to at least one bacterial strain.
- 5 2. Dendritic cells which have been exposed to bacterial species present in the human commensal flora.
3. Dendritic cells as claimed in claim 1 or 2 wherein the bacterial strain is a Lactobacillus.
- 10 4. Dendritic cells as claimed in claim 3 wherein the Lactobacillus is *Lactobacillus salivarius*.
- 15 5. Dendritic cells as claimed in claim 4 wherein the Lactobacillus is *Lactobacillus salivarius* subspecies *salivarius*.
6. Dendritic cells as claimed in any of claims 1 to 5 wherein the bacterial strain is *Lactobacillus salivarius* subspecies *salivarius* 433118.
- 20 7. Dendritic cells as claimed in claim 1 or 2 wherein the bacterial strain is a Bifidobacterium.
8. Dendritic cells as claimed in claim 7 wherein the bacterial strain is *Bifidobacterium infantis*.
- 25 9. Dendritic cells as claimed in claim 7 or 8 wherein the bacterial strain is *Bifidobacterium infantis* 35624.

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10. Dendritic cells as claimed in claim 1 or 2 wherein the bacterial strain is salmonella.
- 5 11. Dendritic cells as claimed in claim 10 wherein the bacterial strain is *Salmonella typhimurium*.
12. Dendritic cells as claimed in claim 10 or 11 wherein the bacterial strain is *Salmonella typhimurium* UK1.
- 10 13. Dendritic cells as claimed in any preceding claim exposed to dead bacteria, or components or mutants thereof.
14. An active derivative, fragment or mutant of dendritic cells as claimed in any preceding claim.
- 15 15. A formulation comprising dendritic cells as claimed in any of claims 1 to 13 or an active derivative, fragment or mutant thereof.
16. A pharmaceutical comprising dendritic cells as claimed in any of claims 1 to 13 or an active derivative, fragment or mutant thereof.
- 20 17. A vaccine comprising dendritic cells as claimed in any of claims 1 to 13 or an active derivative, fragment or mutant thereof.
- 25 18. A method for activating dendritic cells comprising exposing dendritic cells to at least one bacterial strain.
19. A method for activating dendritic cells comprising exposing dendritic cells to bacterial species present in the human commensal flora.

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20. A method as claimed in claim 18 or 19 wherein the bacterial strain is a *Lactobacillus*.
- 5 21. A method as claimed in claim 20 wherein the *Lactobacillus* is *Lactobacillus salivarius*.
22. A method as claimed in claim 21 wherein the *Lactobacillus* is *Lactobacillus salivarius* subspecies *salivarius*.
- 10 23. A method as claimed in any of claims 18 to 22 wherein the bacterial strain is *Lactobacillus salivarius* subspecies *salivarius* 433118.
- 15 24. A method as claimed in claim 18 or 19 wherein the bacterial strain is a *Bifidobacterium*.
25. A method as claimed in claim 24 wherein the bacterial strain is *Bifidobacterium infantis*.
- 20 26. A method as claimed in claim 24 or 25 wherein the bacterial strain is *Bifidobacterium infantis* 35624.
27. A method as claimed in claim 18 or 19 wherein the bacterial strain is *salmonella*.
- 25 28. A method as claimed in claim 27 wherein the bacterial strain is *Salmonella typhimurium*.

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29. A method as claimed in claim 27 or 28 wherein the bacterial strain is *Salmonella typhimurium* UK1.
- 5 30. Dendritic cells whenever activated by a method as claimed in any of claims 18 to 29.
- 10 31. Use of dendritic cells as claimed in any of claims 1 to 13 or 30 or an active derivative fragment or mutant thereof in the prevention and/or treatment of inflammatory disorders, immunodeficiency, inflammatory bowel disease, irritable bowel syndrome, cancer (particularly of the gastrointestinal and immune systems), diarrhoeal disease, antibiotic associated diarrhoea, paediatric diarrhoea, appendicitis, autoimmune disorders, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, coeliac disease, diabetes mellitus, 15 organ transplantation, bacterial infections, viral infections, fungal infections, periodontal disease, urogenital disease, sexually transmitted disease, HIV infection, HIV replication, HIV associated diarrhoea, surgical associated trauma, surgical-induced metastatic disease, sepsis, weight loss, anorexia, fever control, cachexia, wound healing, ulcers, gut barrier function, allergy, 20 asthma, respiratory disorders, circulatory disorders, coronary heart disease, anaemia, disorders of the blood coagulation system, renal disease, disorders of the central nervous system, hepatic disease, ischaemia, nutritional disorders, osteoporosis, endocrine disorders, epidermal disorders, psoriasis and/or acne vulgaris.
- 25 32. A method for the prophylaxis and/or treatment of inflammatory disorders, immunodeficiency, inflammatory bowel disease, irritable bowel syndrome, cancer (particularly of the gastrointestinal and immune systems), diarrhoeal disease, antibiotic associated diarrhoea, paediatric diarrhoea, appendicitis,

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5 autoimmune disorders, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, coeliac disease, diabetes mellitus, organ transplantation, bacterial infections, viral infections, fungal infections, periodontal disease, urogenital disease, sexually transmitted disease, HIV infection, HIV replication, HIV associated diarrhoea, surgical associated trauma, surgical-induced metastatic disease, sepsis, weight loss, anorexia, fever control, cachexia, wound healing, ulcers, gut barrier function, allergy, asthma, respiratory disorders, circulatory disorders, coronary heart disease, anaemia, disorders of the blood coagulation system, renal disease, disorders of the central nervous system, hepatic
10 disease, ischaemia, nutritional disorders, osteoporosis, endocrine disorders, epidermal disorders, psoriasis and/or acne vulgaris comprising administering dendritic cells as claimed in any of claims 1 to 13 or 30 or an active derivative, fragment or mutant thereof.

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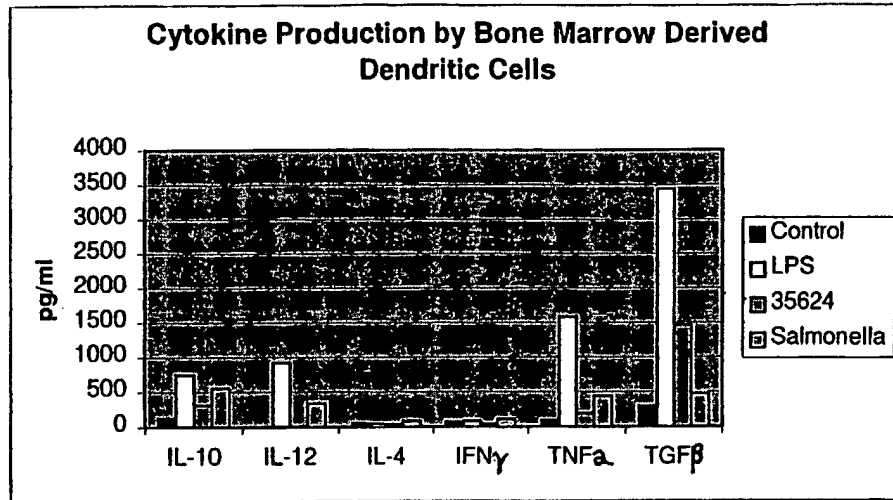


Fig. 1.

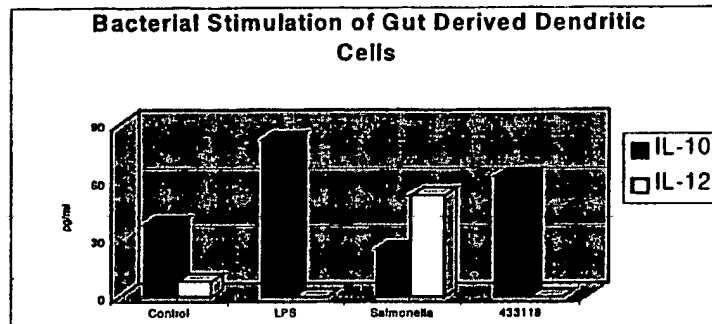


Fig. 2.

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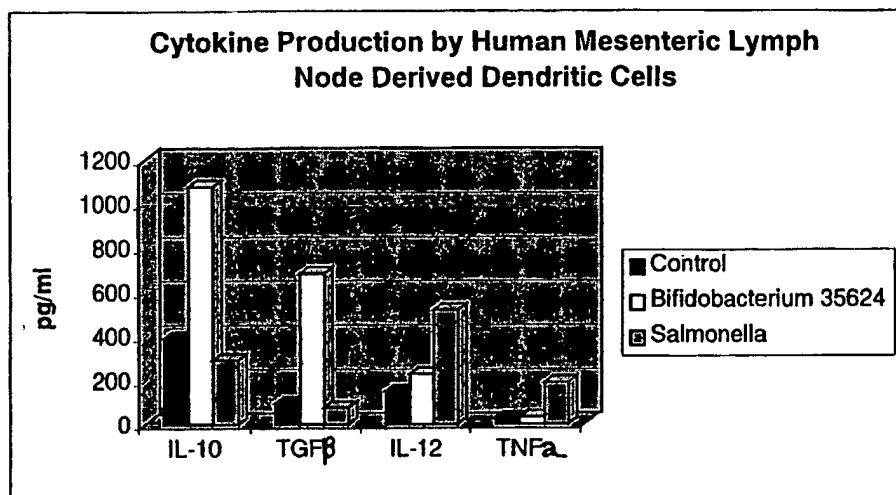


Fig. 3.

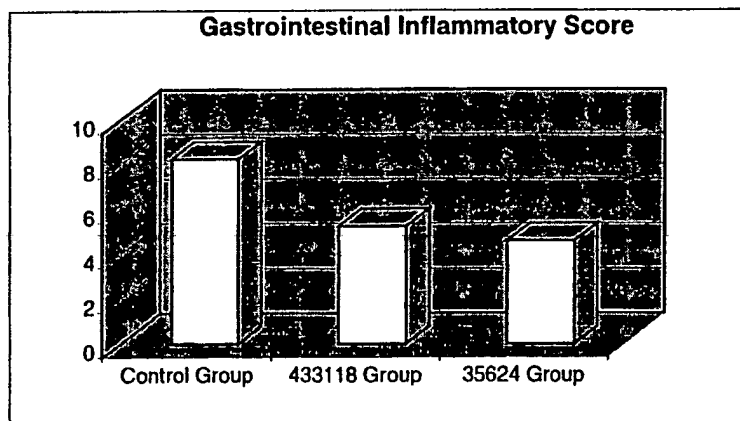


Fig. 4.

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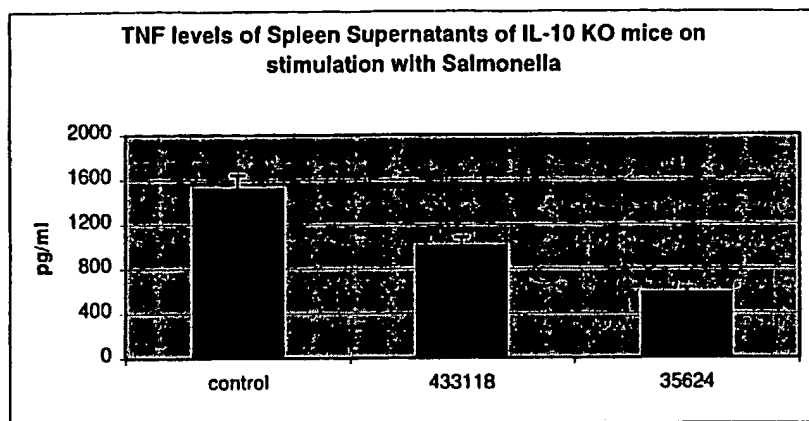


Fig. 5.

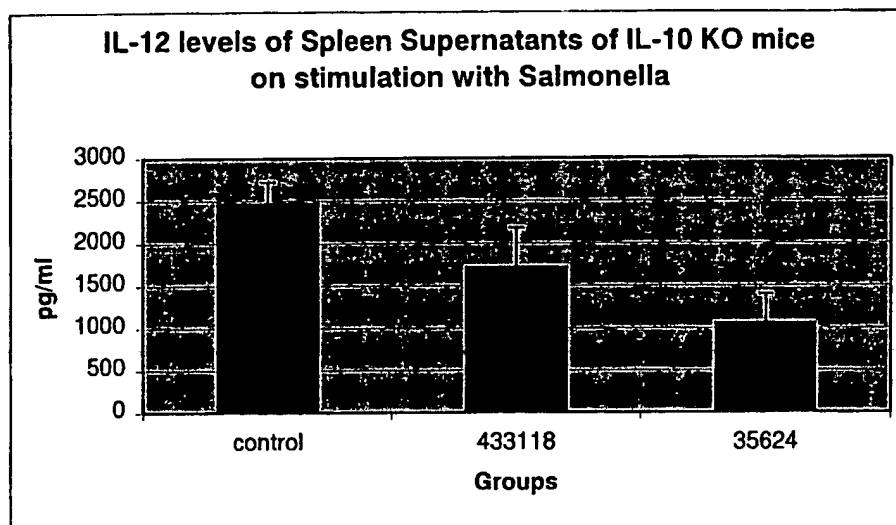


Fig. 6.

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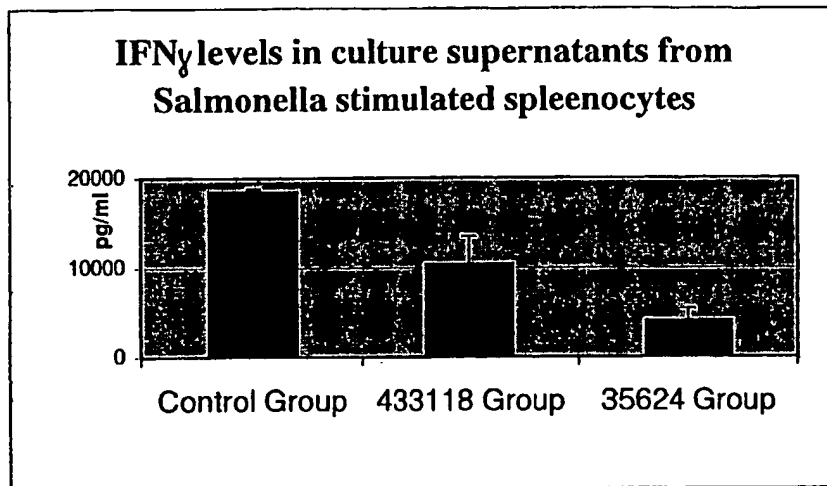


Fig. 7.

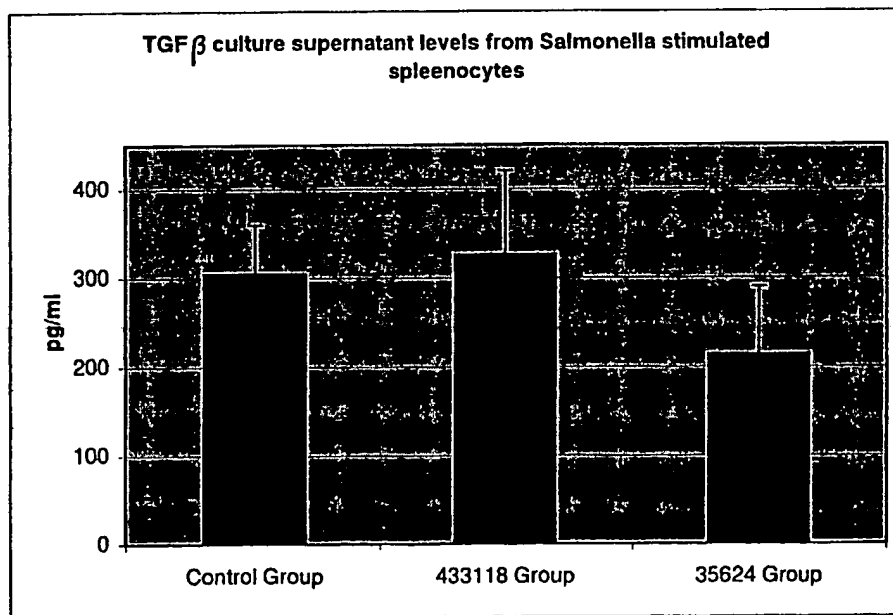


Fig. 8.

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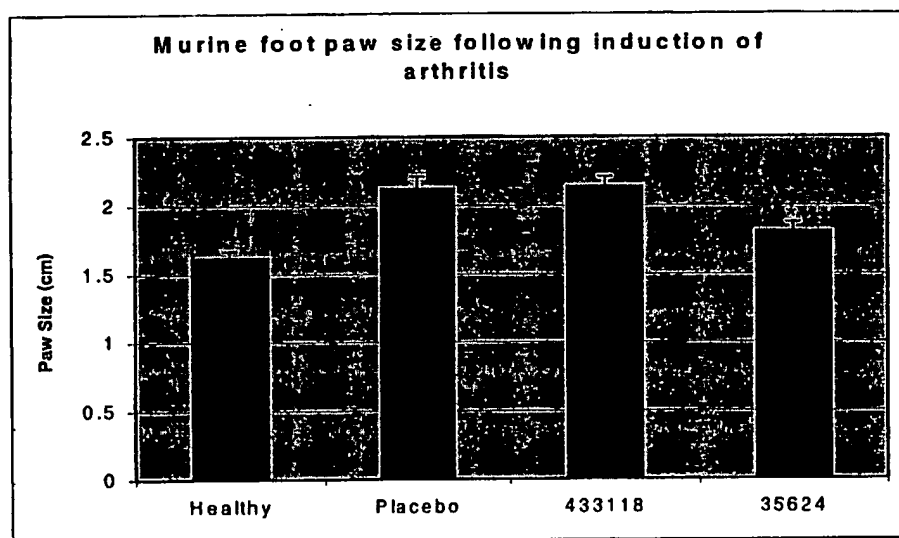


Fig. 9.

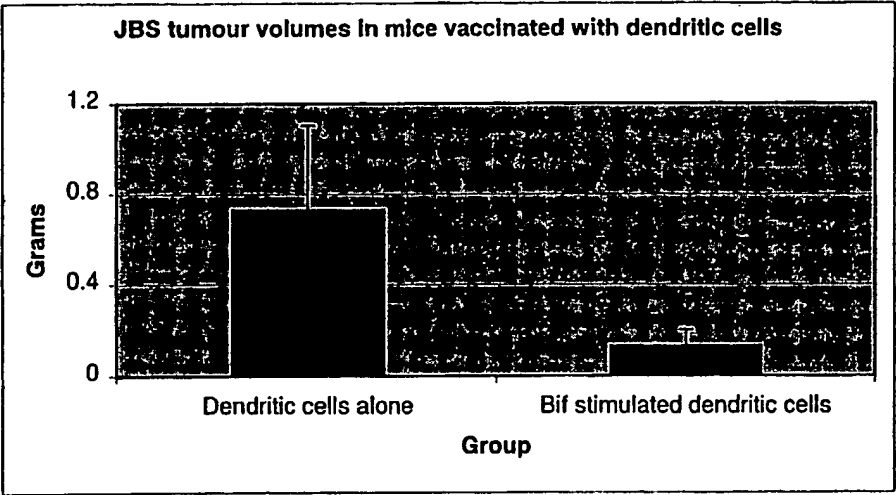


Fig 10.

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(74) Agents: **O'BRIEN, John, A.** et al.; John A. O'Brien & Associates, Third Floor, Duncairn House, 14 Carysfort Avenue, Blackrock, County Dublin (IE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

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Published:

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(88) Date of publication of the international search report:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: IMMUNOTHERAPY BASED ON DENDRITIC CELLS

(57) Abstract: Dendritic cells are exposed to at least one bacterial strain in particular bacterial species present in the human commensal flora. The bacterial strain may be a Lactobacillus and/or Bifidobacterium and/or salmonella strain. The exposed dendritic cells or a formulation, pharmaceutical or vaccine comprising such dendritic cells may be used in the prevention and/or treatment of various diseases such as inflammatory diseases.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IE 02/00043

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 C12N5/08 A61K35/14 A61K35/28 A61K39/02
 A61P1/00 A61P7/00 A61P9/10 A61P17/10 A61P19/02
 A61P25/28 A61P29/00 A61P31/00 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEMANGEL CAROLINE ET AL: "Interaction of dendritic cells with mycobacteria: Where the action starts." IMMUNOLOGY AND CELL BIOLOGY, vol. 78, no. 4, August 2000 (2000-08), pages 318-324, XP002215021 ISSN: 0818-9641 the whole document --- -/--	1,16,17, 31,32



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

27 September 2002

Date of mailing of the international search report

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Moreau, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IE 02/00043

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LINK HANS ET AL: "Vaccination with autologous dendritic cells: From experimental autoimmune encephalomyelitis to multiple sclerosis." JOURNAL OF NEUROIMMUNOLOGY, vol. 114, no. 1-2, 1 March 2001 (2001-03-01), pages 1-7, XP002215026 ISSN: 0165-5728 the whole document</p>	1-32
A	<p>MEDAGLINI D ET AL: "Vaginal immunization with recombinant gram-positive bacteria." ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR, vol. 98, 1998, page 250 XP002215022 98th General Meeting of the American Society for Microbiology; Atlanta, Georgia, USA; May 17-21, 1998, 1998 ISSN: 1060-2011 the whole document</p>	1-32
A	<p>HUANG Y -M ET AL: "Autoantigen-pulsed dendritic cells induce tolerance to experimental allergic encephalomyelitis (EAE) in Lewis rats." CLINICAL AND EXPERIMENTAL IMMUNOLOGY, vol. 122, no. 3, December 2000 (2000-12), pages 437-444, XP002215023 ISSN: 0009-9104 the whole document</p>	1-32
A	<p>WO 98 35014 A (BIORESEARCH IRELAND) 13 August 1998 (1998-08-13) cited in the application the whole document</p>	1-32
A	<p>WO 00 42168 A (ENTERPRISE IRELAND) 20 July 2000 (2000-07-20) the whole document</p>	1-32
P,X	<p>O'MAHONY LIAM ET AL: "Probiotic bacteria and pathogenic bacteria elicit differential cytokine responses from dendritic cells." GASTROENTEROLOGY, vol. 120, no. 5 Supplement 1, April 2001 (2001-04), page A.315 XP002215024 102nd Annual Meeting of the American Gastroenterological Association and Digestive Disease Week; Atlanta, Georgia, USA; May 20-23, 2001, April, 2001 ISSN: 0016-5085 the whole document</p>	1-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IE 02/00043

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 32 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
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Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IE 02/00043

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9835014	A	13-08-1998	AU 748744 B2	13-06-2002
			EP 1002051 A2	24-05-2000
			WO 9835014 A2	13-08-1998
			JP 2002514068 T	14-05-2002
			NO 993818 A	11-10-1999
			NZ 337505 A	27-07-2001
WO 0042168	A	20-07-2000	AU 3071500 A	01-08-2000
			AU 3071600 A	01-08-2000
			AU 3071700 A	01-08-2000
			BR 0007481 A	09-04-2002
			BR 0007550 A	30-10-2001
			CN 1338940 T	06-03-2002
			CN 1342196 T	27-03-2002
			EP 1143985 A2	17-10-2001
			EP 1141235 A2	10-10-2001
			EP 1145001 A2	17-10-2001
			WO 0041707 A2	20-07-2000
			WO 0042168 A2	20-07-2000
			WO 0042429 A2	20-07-2000
			NO 20013429 A	27-08-2001
			NO 20013467 A	14-09-2001
			TR 200102058 T2	21-12-2001
			TR 200102059 T2	21-02-2002
			US 2002006432 A1	17-01-2002

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